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MINIREVIEW

Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells

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A wide variety of pathogenic microorganisms have been demonstrated to cause eukaryotic cell death, either as a consequence of infecting host cells or by producing toxic products. Pathogen-induced host cell death has been characterized as apoptosis in many of these systems. It is increasingly being recognized that cell death with some of the features of apoptosis may result from a variety of molecular pathways and that experimental techniques used to identify cell death often do not distinguish among these mechanisms. We propose that a clear understanding of the diversity of processes mediating cell death has been obscured by the simplicity of the nomenclature system commonly employed to describe eukaryotic cell death. This review presents a perspective on eukaryotic cell death and discusses experimental techniques used to study these processes.

SIGNIFICANCE OF HOST CELL DEATH IN INFECTION

Perhaps the most obvious potential outcome of host-pathogen interactions is the death of host cells, and this has long been known to result from infection (49). The study of pathogen-induced host cell death has gained attention with the recognition that this phenomenon may not be merely an incidental finding during infection but, rather, a controlled and modifiable process with significant implications for disease pathogenesis (37). Host cell death may impair normal organ function and lead to associated signs and symptoms of disease. Microbial pathogens may improve their ability to persist in infected hosts by causing the death of cells required for host defense (147). Although some intracellular pathogens may employ strategies to prevent cell death during pathogen replication, escape and dissemination to new host cells may eventually require cell lysis.

Pathogen-induced cell death, a seemingly simple outcome, may occur by a variety of complex mechanisms. Elucidating the factors required by a pathogen to kill host cells is, therefore, critical to uncovering mechanisms of pathogenesis. Understanding the process of dying may reveal why certain cells may be more or less susceptible to pathogen-induced cell death and reveal novel therapeutic targets. Furthermore, the mechanism of cell death may have significant consequences in terms of the ensuing response to the dead cell by modulating inflammation or influencing the immune response (1, 112). Additionally, studies regarding the processes leading to pathogen-induced cell death are likely to shed light on the mechanisms of cell death occurring during other physiological and pathological processes.

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APOPTOSIS AND NECROSIS PARADIGM

Cell death is typically discussed dichotomously as either apoptosis or necrosis. Apoptosis is described as an active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation. Necrosis has been characterized as passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents. As apoptosis is considered to be a regulated and controlled process, its occurrence during particular infectious processes has received great attention.

A number of pathogens have been described to cause host cell death with features of apoptosis (for reviews, see references 37, 42, 92, and 138). Some pathogenic bacteria secrete pore-forming toxins or protein synthesis inhibitors, which have been associated with host cell apoptosis (92). Multiple viral proteins are reported to induce apoptosis (42). In addition, several parasites and pathogenic yeasts have been identified as mediators of apoptosis (39, 55, 92). These are not simply observations confined to cell culture. Pathogen-induced apoptosis has also been described in tissues of animals infected with pathogens such as *Listeria monocytogenes* (104), *Mycobacterium tuberculosis* (137), and *Yersinia pseudotuberculosis* (90). Although it is assumed that all pathogen-induced deaths that have been characterized as apoptosis truly converge on final common pathways that result in equivalent postmortem outcomes, such as apoptotic body removal and inhibition of inflammation, this assumption remains unexplored.

Despite the widespread use of the apoptosis-versus-necrosis paradigm, there is an increasing awareness of the complexity of processes occurring in dying cells that lead to the outcome of death. Below, we highlight advances in the study of cell death and suggest approaches for experimental interpretation. As biology does not necessarily conform to the simple paradigms created by our existing terminology, another goal is to develop nomenclature to accurately describe and distinguish pathways of cell death. It will be useful to begin by tracing the main developments that led us to where we now stand.

APOPTOSIS

The term apoptosis was proposed by Kerr and colleagues in 1972 to describe a specific morphological pattern of cell death observed as cells were eliminated during embryonic development, normal cell turnover in healthy adult tissue, and atrophy upon hormone withdrawal (57). The morphology associated with this phenomenon was characterized by nuclear and cytoplasmic condensation and cellular fragmentation into membrane-bound fragments. These fragments or apoptotic bodies were taken up by other cells and degraded within phagosomes. The authors suggested that the deletion of cells with little tissue disruption and no inflammation allows reutilization of cellular components. The morphological characteristics of apoptosis were proposed to result from a general mechanism of controlled cell deletion, which plays a complementary role to mitosis and cytokinesis in maintaining stable cell populations within tissues. The concept of apoptosis furthered the hypothesis (76, 78) that living cells are genetically programmed to contain components of a metabolic cascade that, when activated, can lead to cellular demise.

The word apoptosis was used in Greek to denote a "falling off," as leaves from a tree (57). The term connotes a controlled physiologic process of removing individual components of an organism without destruction or damage to the organism. To show the derivation clearly, the authors proposed that the stress should be on the penultimate syllable, with the second half of the word being pronounced like "ptosis" with a silent "p," which comes from the same root "to fall" and is used in medicine to describe drooping of the upper eyelid.

This landmark paper first proposed that cell death resulting from intrinsic cellular processes should be considered distinctly different from cell death caused by severe environmental perturbations. The latter process was associated with the morphology of coagulation necrosis which "is probably the result of an irreversible disturbance of cellular homeostatic mechanisms" (57).

The developmental timing and consistent morphological pattern associated with apoptosis suggested the genetic basis of this program of cell death. Characterization of *Caenorhabditis elegans ced* (cell death abnormal) mutants revealed gene products involved in cell death during embryonic development (43). The amino acid sequence of CED-3 shows similarity to a mammalian protease known as interleukin-1β (IL-1β)-converting enzyme (2, 144). Subsequent investigation revealed the existence of a family of these proteases, now known as caspases or cysteine-dependent aspartate specific proteases, and IL-1β-converting enzyme was renamed caspase-1 (2). Caspases exist as latent zymogens that contain an N-terminal prodomain followed by the region that forms a two-subunit catalytic effector domain (135, 140).

Although all members of the caspase family share similarities in amino acid sequence and structure, they differ significantly in their physiologic roles (Fig. 1). The caspases can be broadly divided into two groups: those that are centrally involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) and those related to caspase-1 (caspase-1, -4, -5, -13, and -14, as well as murine caspase-11 and -12), whose primary role appears to be in cytokine processing during inflammatory responses (20). The caspases implicated in apoptosis can be further divided into two subgroups based on their structure and the temporal aspects of their activation during cell death (79). Initiator caspases (caspase-2, -8, -9, and -10) have long prodomains and are primarily responsible for initiating caspase activation cascades. Effector caspases (caspase-3, -6, and -7) generally contain only a small prodomain and are responsible for the actual dismantling of the cell by cleaving cellular substrates. Activation of initiator caspases requires dimerization, which is mediated by binding of their prodomains to adaptor molecules via caspase recruitment domain or death effector domain motifs (6). Upon activation, initiator caspases propagate death signals by activating downstream effector caspases in a cascade-like manner (120). Effector caspases are converted into their active forms through proteolysis at internal Asp residues, allowing the assembly of active heterotetramers composed of two large subunits and two small subunits (6). Infectious pathogens may co-opt caspase activation domains to induce host cell death. For example, *Chlamydia trachomatis* produces a protein, called *Chlamydia* protein associating with death domains, that interacts with the death domains of tumor necrosis factor family receptors to activate apoptotic caspases (123).

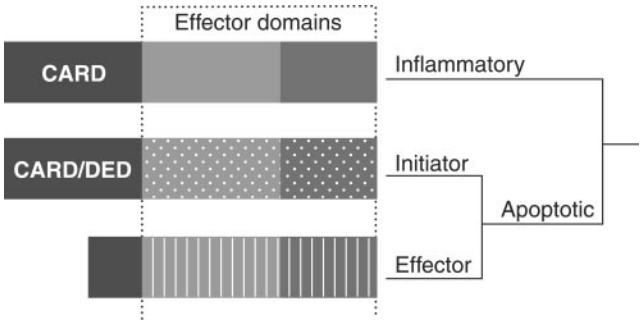

FIG. 1. Caspases are classified into functional subgroups. Caspases are cysteine proteases that are expressed as inactive precursor enzymes with an N-terminal prodomain followed by a two-subunit effector domain. Members of the caspase family can be classified based on their physiologic roles and substrate specificities. They are divided into two main groups: those involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) and those related to caspase-1 (caspase-1, -4, -5, -13, and -14, as well as murine caspase-11 and -12), whose primary role appears to be cytokine processing and proinflammatory cell death. The caspases implicated in apoptosis can be further divided into initiator and effector subgroups. Initiator caspases (caspase-2, -8, -9, and -10) have long prodomains and function to activate effector caspases (caspase-3, -6, and -7), which have small prodomains and cleave a variety of cellular substrates. CARD, caspase recruitment domain; DED, death effector domain.

Activated effector caspases selectively cleave a restricted set of target proteins to produce the morphological and biochemical features associated with apoptosis (Fig. 2). One often used marker of apoptosis is the DNA ladder produced by cleavage of genomic DNA between nucleosomes to generate fragments with lengths corresponding to multiple integers of approximately 180 base pairs (141). The nuclease responsible for this characteristic, caspase-activated DNase (CAD; also named DFF-40) is present in living cells bound to its inhibitor (inhibitor of CAD [ICAD], also named DFF-45). Activation of CAD occurs via cleavage of ICAD mediated by caspase-3 and

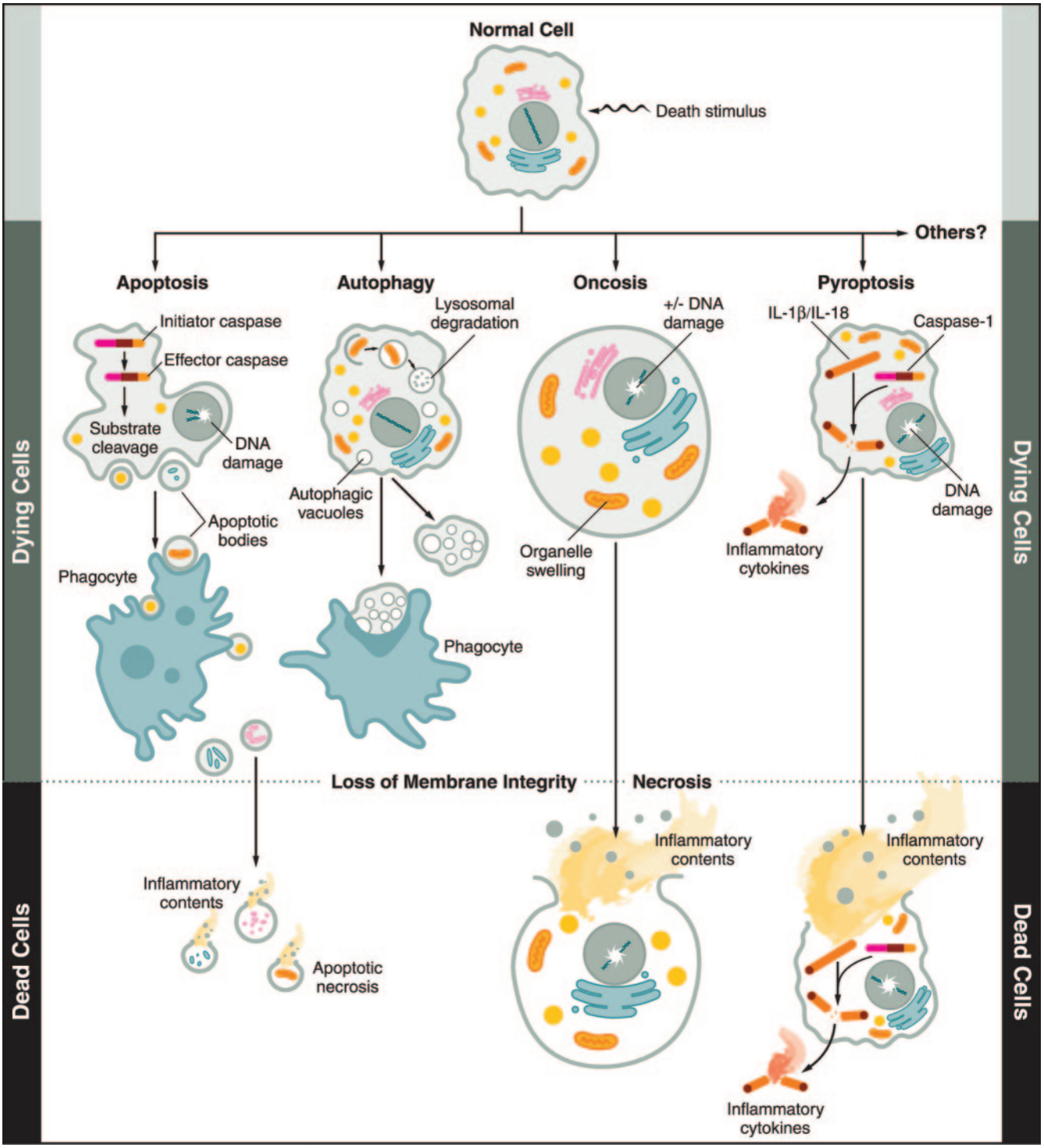

FIG. 2. Pathways leading to cell death. Healthy cells respond to death-inducing stimuli by initiating a variety of molecular pathways leading to cell death. Completion of the proper pathway is a critical cellular function to ensure that the appropriate outcome is ultimately achieved in a multicellular organism. Failure to die in response to particular stimuli can result in abortive embryogenesis and organ dysfunction and contributes to the initiation of cancer. Proinflammatory death is vital in triggering appropriate immune responses or, in the extreme, may cause tissue pathology and organ dysfunction. Therefore, pathway utilization can dramatically influence biological systems. Apoptosis is a pathway leading to cell death that features the activation of initiator caspases that activate effector caspases to cleave cellular substrates. Apoptotic cells demonstrate cytoplasmic and nuclear condensation, DNA damage, formation of apoptotic bodies, maintenance of an intact plasma membrane, and exposure of surface molecules targeting intact cell corpses for phagocytosis. In the absence of phagocytosis, apoptotic bodies may proceed to lysis and secondary or apoptotic necrosis. Autophagy features degradation of cellular components within the intact dying cell in autophagic vacuoles. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. Autophagic cells can also be taken up by phagocytosis. Oncosis is the prelethal pathway leading to cell death accompanied by cellular and organelle swelling and membrane breakdown, with the eventual release of inflammatory cellular contents. Pyroptosis is a pathway to cell death mediated by the activation of caspase-1, a protease that also activates the inflammatory cytokines, IL-1β, and IL-18. This pathway is therefore inherently proinflammatory. Pyroptosis also features cell lysis and release of inflammatory cellular contents. Undoubtedly, other pathways exist that have not yet been described.

caspase-7, resulting in the release and activation of CAD (29, 74, 106).

Caspase proteolysis of additional substrates explains the other morphological changes initially used to describe apoptosis. Lamins, the scaffold proteins of the nuclear envelope, are cleaved by effector caspases, leading to nuclear shrinkage and fragmentation (10, 64, 103). Loss of overall cell shape is probably caused by the cleavage of cytoskeleton proteins such as fodrin (62). Cleavage of the components of the focal adhesion complex leads to detachment of apoptotic cells from their neighbors and the basement membrane (21, 139). Plasma membrane blebbing results from the caspase-mediated activation of gelsolin, an actin depolymerizing enzyme (62). Caspase-mediated cleavage of PAK2, a member of the p21-activated kinase family, participates in the formation of apoptotic bodies (105).

Another caspase-dependent process is phosphatidylserine (PS) exposure. PS is actively localized on the inner leaflet of the plasma membrane in healthy cells. The asymmetry of its distribution is lost in apoptotic cells. PS exposure on the outer leaflet of the plasma membrane can be recognized by phagocytes as a signal for engulfment (32, 131). PS exposure has been reported to be caspase dependent (13, 83), but its mechanism has not been totally elucidated. A combined effect of down-regulation of a phospholipid translocase activity and activation of a lipid scramblase, which are observed in apoptotic lymphocytes, may contribute to PS exposure (91).

Originally, the term apoptosis was defined purely on morphological grounds, and therefore this name has been applied to anything that looks like apoptosis (51, 130). However, as the biochemical mechanisms leading to changes in cell morphology have been discovered, the term apoptosis has become associated with a wide variety of meanings. To simplify, Samali et al. (107) and others (5) have proposed that apoptosis be defined as caspase-mediated cell death with the following morphological features: cytoplasmic and nuclear condensation, chromatin cleavage, formation of apoptotic bodies, maintenance of an intact plasma membrane, and exposure of surface molecules targeting cell corpses for phagocytosis. More specifically, the molecular definition of apoptosis can logically be based on the proteolytic activity of certain caspases (caspase-2, -3, -6, -7, -8, -9, and -10) because these enzymes mediate the process of apoptotic cell death.

NECROSIS

The biological significance and greater appreciation of the enzymatic machinery involved in apoptosis indicate the importance of distinguishing this process from cell death that occurs by other mechanisms. The need for clarity in scientific communication and the goal of constructing informative testable hypotheses bring out a major problem regarding the nomenclature of cell death, which is the lack of suitable names or classifications for cell death that does not occur by apoptosis. Necrosis is the term currently used for nonapoptotic, accidental cell death. However, a key issue that has often been overlooked in the cell death literature is the distinction between the structural and biochemical processes occurring in a dying cell and the endpoint of death itself (34, 56). Necrosis is a term used by pathologists to designate the presence of dead tissues or cells and is the sum of changes that have occurred in cells after they have died, regardless of the prelethal processes (68, 69, 81). Necrosis, therefore, refers to morphological stigmata seen after a cell has already died and reached equilibrium with its surroundings (Fig. 2) (115). Thus, in the absence of phagocytosis, apoptotic bodies may lose their integrity and proceed to secondary or apoptotic necrosis. Here, the term apoptotic necrosis describes dead cells that have reached this state via the apoptotic program (81). The presence of necrosis tells us that a cell has died but not necessarily how death occurred (115).

ONCOSIS

The term oncosis has been accepted by many investigators of cell death as a counterpoint to apoptosis (68, 81, 96). Oncosis (from "onkos," meaning swelling) is defined as a prelethal pathway leading to cell death accompanied by cellular swelling, organelle swelling, blebbing, and increased membrane permeability (Fig. 2) (81). The process of oncosis ultimately leads to depletion of cellular energy stores and failure of the ionic pumps in the plasma membrane. Oncosis may result from toxic agents that interfere with ATP generation or processes that cause uncontrolled cellular energy consumption (81). It is now being recognized that the changes accompanying oncosis may result from active enzyme-catalyzed biochemical processes (128).

For example, poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that is activated by DNA strand breaks to catalyze the addition of poly(ADP-ribose) to a variety of nuclear proteins (102). In situations of moderate DNA damage, this activity of PARP participates in DNA repair (44). However, with massive DNA destruction, excessive PARP activity depletes its substrate NAD. Resynthesis of NAD depletes ATP, and the eventual loss of energy stores leads to oncotic cell death (134). In this way, energy depletion and oncosis occur as a regulated response to severe DNA injury (4). During apoptosis, caspases cleave and inactivate PARP, which preserves cellular ATP despite significant DNA damage (102).

Altered intracellular calcium levels may also regulate oncotic cell death (127). Elevated cytoplasmic calcium concentrations can activate cysteine proteases of the calpain family that mediate plasma membrane breakdown through the proteolysis of cytoskeletal and plasma membrane proteins (72, 73). Increased intracellular calcium also initiates translocation of cytosolic phospholipase A_2s to cellular membranes, where the hydrolysis of membrane phospholipids decreases membrane integrity (22, 109).

Oncosis induced by pathogen infection has been suggested in a number of experimental models. Rotavirus infection of MA104 cells induces cell death morphologically consistent with oncosis, which also requires increased intracellular calcium (99). In addition, *Pseudomonas aeruginosa* infection induces oncosis in infected macrophages and neutrophils (23). These cells demonstrate swelling, rapid plasma membrane breakdown, and swollen nuclei without internucleosomal DNA fragmentation.

AUTOPHAGY

Apoptotic bodies and the cellular debris released during lysis of oncotic cells can both be phagocytized and degraded by neighboring viable cells in vivo (128). Another form of cell

TABLE 1. Relevant terms for describing dead and dying cells

Term	Characteristic(s)
Programmed cell death	Dependent on genetically encoded signals or activities within the dying cell; a sequence of potentially modifiable events leading to the death of the cell
Apoptosis	Mediated by a subset of caspases (Fig. 1); morphology includes nuclear and cytoplasmic condensation and formation of membrane-bound cellular fragments or apoptotic bodies; not inflammatory
Autophagy	Degradation of cellular components within the dying cell in autophagic vacuoles; not inflammatory
Oncosis	Prelethal pathway leading to cell death accompanied by cellular and organelle swelling and increased membrane permeability; proinflammatory
Pyroptosis	Proinflammatory pathway resulting from caspase-1 activity leading to membrane breakdown and proinflammatory cytokine processing
Necrosis	Postmortem observation of dead cells that have come into equilibrium with their environment

death, autophagy or type II cell death, features degradation of cellular components within the dying cell in autophagic vacuoles (16). The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation (Fig. 2) (11). Autophagy has been well described during vertebrate development and may be a phylogenetically old process (12).

Studies on autophagy suggest that it proceeds through a sequence of morphological changes in a highly regulated process (40, 59). Briefly, the autophagic pathway begins with the sequestration of cytoplasmic material in double-membrane vesicles known as autophagosomes (59). The sequestration process is under the control of GTPases (100) and phosphatidylinositol kinases (95) and involves novel ubiquitin-like conjugation systems (97). Autophagosomes then fuse with lysosomes in a process depending on microtubules, and the contents are degraded (59). In vivo, cells undergoing autophagy can be phagocytized by neighboring cells (16, 116).

CONSEQUENCES OF CELL DEATH

The story of cell death in vivo does not end with the completion of the series of molecular events that give rise to cellular demise. Intrinsic to the processes of cell death may be mechanisms that allow cellular corpses to communicate with living cells in surrounding organs and tissues. Apoptotic cells can display a variety of recognition signals for phagocytes (112) that lead to their swift removal. Recent evidence further suggests that some apoptotic cells may secrete chemotactic factors that cause local accumulation of macrophages (63, 114). Cells undergoing autophagy are also taken up by phagocytosis (16, 116). Oncotic cells, however, proceed to necrosis with lysis and spillage of cellular contents before the dying cells can be recognized by phagocytes (111). The released contents of necrotic cells include molecules that act as signals to promote inflammation (113, 118). In contrast, the uptake of apoptotic bodies suppresses secretion of inflammatory mediators from activated macrophages (31). Therefore, a critical component of the definitions of apoptosis and autophagy is their anti-inflammatory outcome. Furthermore, an essential element of oncosis is its inflammatory nature. Appreciating these potential consequences of pathogen-induced host cell death may be essential both for optimal vaccine design and understanding the persistence and pathogenesis of infections and inflammatory conditions.

PYROPTOSIS

Along with other investigators, we have begun characterizing a novel form of cell death induced by infection with *Salmonella* and *Shigella* species that is inherently proinflammatory (7, 19, 82, 94, 108, 146). This pathway of cell death is uniquely dependent on caspase-1 (Fig. 2) (9, 15, 46–48). Caspase-1 is not involved in apoptotic cell death and caspase-1-deficient cells respond normally to most apoptotic signals (71). An important function of caspase-1 is to process the proforms of the inflammatory cytokines, IL-1β and IL-18, to their active forms (33). Caspase-1 activation in macrophages infected with *Salmonella* or *Shigella* results in processing of these cytokines and death of the host cell (46, 88, 94, 146). The mechanism and outcome of this form of cell death are distinctly different from these aspects of apoptosis, which actively inhibits inflammation. We have proposed the term pyroptosis from the Greek roots "pyro," relating to fire or fever, and "ptosis" (pronounced "to-sis"), denoting falling, to describe proinflammatory programmed cell death (19). The observed caspase-1 activation or dependence during cell death in the immune (117), central nervous (75, 145), and cardiovascular systems (36, 60) indicates that pyroptosis plays a significant role in a variety of biological systems.

PROGRAMMED CELL DEATH

Apoptosis and programmed cell death are often used as synonyms. However, as we have discussed, a variety of other molecular cell death pathways have been characterized. Programmed cell death may be more accurately defined as cell death that is dependent on genetically encoded signals or activities within the dying cell (Table 1) (54, 78). Therefore, the designation "programmed" refers to the fixed pathway followed by dying cells, regardless of the mechanism (Fig. 2) or of whether the characteristic features of apoptosis accompany the process. Acute cell breakdown due to the direct action of a damaging stimulus is the conceptual converse of programmed cell death since it requires no cellular activity and is prevented

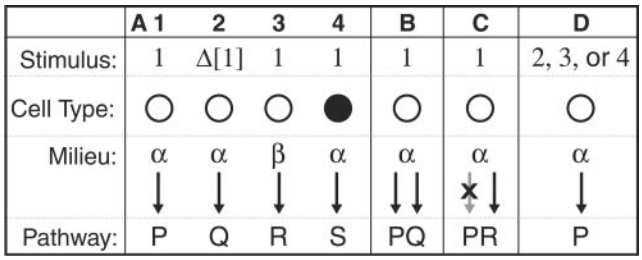

FIG. 3. Biological input differentially influences utilization of cell death pathways. Factors influencing cell death include the stimulus, cell type, and the surrounding milieu of the cell and, therefore, its physiological state during receipt of the stimulus, which together dictate the pathway of cell death. In column A1, a stimulus (1) delivered to a cell type of interest in its baseline physiological state (α) dictates cell death via the primary pathway (P). Altering the magnitude of the stimulus (Δ1), e.g., multiplicity of infection, concentration, or concentration per unit time, can alter the path to cell death (A2, leading to pathway Q), as does the physiological state (β) of the cell during stimulation (A3, leading to pathway R) and the cell type being studied (A4, leading to pathway S). A single stimulus can simultaneously trigger multiple pathways (column B), and blockade of the primary pathway for a given cell type (column C versus column A1) can result in the use of alternate pathway(s) (column C). Finally, multiple stimuli (2,3, or 4), which may utilize a variety of upstream signaling cascades, may converge in the use of biologically conserved effector pathways and result in cell death (column D).

only by the absence of the damaging stimulus (54). Autophagy has been well documented as a mechanism of programmed cell death occurring during the process of normal embryonic development (16, 70). The dependence of pyroptosis on the activation of caspase-1 also indicates that it is a program of cell death (7, 19). Furthermore, increasing genetic data indicate that oncosis requires an intrinsic molecular program (80, 87).

COMPLEXITY OF CELL DEATH

Thus far, we have described modes of cell death as discrete, independent entities. However, recent observations do not support strict paradigmatic distinctions between these designations in some biological systems but, instead, suggest the overlapping nature of the processes occurring in dying cells (Fig. 3). Multiple types of death can be observed simultaneously in tissues or cell cultures exposed to the same stimulus (3, 61, 119). The local intensity of a particular initial insult may influence the mechanism of demise taken by individual cells in a population (8, 27, 35, 148). For example, small numbers of *Escherichia coli* have been shown to inhibit polymorphonuclear neutrophil apoptosis, whereas larger numbers of *E. coli* promote polymorphonuclear neutrophil death (84). Furthermore, the activation state or differentiation state of individual cells may determine the dominant death pathway invoked by a particular stimulus. *Shigella flexneri* induces cell death with features of apoptosis in gamma interferon-differentiated U937 cells, whereas death of undifferentiated or retinoic acid-differentiated U937 cells has features most consistent with oncosis (93).

Recent evidence suggests that multiple pathways may be activated in single dying cells and cross talk between cell death programs may allow fine control over the ultimate outcome (67, 77). Inhibiting the dominant molecular route of cell death may not result in survival but, rather, allow the occurrence of alternate programs leading to different types of cell death. For example, *Salmonella* infection of caspase-1-deficient macrophages bypasses pyroptosis but results in a form of delayed cell death with features of autophagy (45, 52). It has also been demonstrated in a variety of systems that stimuli initiating caspase-dependent apoptosis will cause cell death, albeit by a different mechanism, even in the presence of caspase inhibitors (54, 66). Caspase inhibition in vivo may result from S-nitrosylation caused by endogenously produced nitric oxide (25, 58, 86) or viral caspase inhibitors such as the cowpox inhibitor CrmA (124). The characteristics of cell death observed in such situations often bear limited homology to apoptosis and instead appear similar to those of oncosis (50, 110, 142). For example, caspase inhibition in *Staphylococcus aureus* alpha-toxin-treated cells or human immunodeficiency virus type 1-infected cells prevents internucleosomal DNA fragmentation but not the eventual loss of membrane integrity (30, 101). These findings suggest that a single stimulus can initiate multiple distinct modes of cell death and that cellular physiological states determine the ultimate outcome in response to a particular stimulus.

As we have discussed, the molecular processes that mediate cell death are more complicated than may have been initially appreciated. Recent advances have enhanced our understanding of the subtleties underlying cell death, but many forms of cell death may remain incompletely characterized. In addition to those we have discussed here, other cell death programs including paraptosis are being described (14, 121, 122). We propose that novel physiologically and pathologically relevant pathways of cell death with unique features and implications await discovery and delineation. Studies of host-pathogen interactions have revealed many fundamental features of basic eukaryotic biology such as factors mediating the dynamic aspects of the cytoskeleton. Examining death pathways evoked by pathogens may lead to the characterization of novel forms of cell death and elucidation of unidentified pathways of executing cellular demise. Our final classification of such deaths, it is hoped, will be determined by the molecular pathways that are activated in the dying cell and the postmortem consequences that result from particular types of cell death. This will require experiments and interpretations aimed at characterizing unique features of novel forms of cell death.

EXPERIMENTAL METHODS USED IN THE STUDY OF CELL DEATH

A variety of techniques and reagents have been developed to study cell death, each with particular advantages and limitations. Many methods are purported to identify apoptosis; however, most of these techniques alone are not sufficient to prove that apoptosis, but not another form of cell death, has occurred. It should also be emphasized that experimentally we define cell death based on measurements of particular characteristics associated with cell death. For clarity, representing experimental data in terms of the feature of death measured is preferable to simply reporting percent apoptosis or percent death. Different features of death may not necessarily be functionally related and may occur via distinct mechanisms elicited by a single initiating stimulus. For example, DNA fragmenta-

tion and apoptotic body formation occur via distinct processes, but both depend on caspases (105, 106). Although inhibiting particular aspects of cell death may not prevent the eventual demise of cells, the particular features of cell death may endow dying cells with important functional consequences. For example, PS exposure may not be a requisite step in the pathway to death but may be required for the appropriate noninflammatory outcome of apoptotic cell death.

The study of apoptosis was initially based on cell morphology by using light microscopy and electron microscopy to identify nuclear and cytoplasmic condensation and cellular fragmentation (57). The identification of morphological changes occurring in dying cells by light or electron microscopy is certainly useful in the characterization of pathogen-induced cell death. A variety of standard histological stains or fluorescent dyes can be used to demonstrate condensed chromatin (85). However, oncotic cells can also have condensed chromatin (18), and thus a caveat of visual inspection is the difficulty in distinguishing apoptotic from oncotic cells (85). Furthermore, visualizing particular morphological features suggests, but falls short of demonstrating, the underlying biochemical processes.

Detection of DNA fragmentation is currently one of the most frequently used techniques in the study of cell death. Internucleosomal DNA fragmentation can be visualized by gel electrophoresis as the characteristic DNA ladder and was previously considered the biochemical hallmark of apoptosis. Highly sensitive cytochemical techniques have been developed to visualize DNA fragmentation in individual nuclei. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method utilizes the activity of the terminal deoxynucleotidyl transferase enzyme to label the 3′ ends of DNA strand breaks, which may then be identified in individual nuclei by microscopy. However, necrotic cells can exhibit both DNA laddering (17, 136) and TUNEL-positive nuclei as well (24, 26, 41). Thus, while DNA degradation is an important event in cell death (Fig. 2), its detection in dead cells does not specifically indicate the underlying mechanism of death.

The loss of structural integrity of the plasma membrane is a hallmark of necrosis and represents the common final endpoint at which a cell can no longer maintain its discrete identity from the environment. By definition, this is manifested biochemically as the release of cytosolic enzymes including lactate dehydrogenase and uptake of membrane-impermeant dyes such as trypan blue, propidium iodide, and 7-aminoactinomycin D (85). Though membrane destruction is a universal indication of cell death, it does not imply the antecedent mechanisms leading to death.

Translocation of PS to the outer surface of the plasma membrane serves as a recognition signal for phagocytosis of dying cells (32). Loss of phospholipid asymmetry is detected experimentally with the use of annexin V, which specifically binds PS and can be detected by flow cytometry when fluorescently conjugated (129). The externalization of PS is an early event of apoptosis, occurring while the plasma membrane remains intact and cells exclude membrane-impermeant dyes. Examples of PS exposure prior to membrane compromise have also been observed in oncotic cells, and this may not necessarily be a feature unique to apoptosis (65). Necrotic cells display annexin V staining concurrently with vital dye uptake, indicating that annexin V binding is the result of membrane damage. Therefore, a critical control that must be included is to demonstrate the membrane integrity of PS-positive cells, i.e., exclusion of membrane-impermeant dyes.

A more specific and comprehensive understanding of the mechanisms responsible for cell death requires delineation of the individual enzymatic and biochemical steps of execution. Caspase activity can be demonstrated by Western blotting by using specific antibodies against caspase substrates such as PARP and lamins (85, 125). In addition, proteolytic cleavage and activation of effector caspases can be observed by Western blotting by using antisera against caspases themselves (53). Caspase activity can also be measured by using colorimetric and fluorometric assays based on proteolysis of conjugated tetrapeptide substrates mimicking caspase cleavage sites (98). These techniques have the disadvantage of measuring enzyme activity in a cell population and not in individual cells. This limitation is overcome by staining cells with antibodies specific for processed caspases and by using flow cytometry to interrogate individual cells (132). Peptide-conjugated caspase inhibitors can be used to determine the requirement for particular caspases in cell death processes. While these peptide-based inhibitors were designed to mimic optimal caspase cleavage motifs, some degree of overlap in caspase activity (126) can lead to nonspecific inhibition (28, 133). However, particular inhibitors have a great deal of specificity for their targets (38). For example, Ac-YVAD-CHO exhibits a K_i over 200-fold lower for caspase-1 than for any other caspase tested, and this degree of selectivity makes this inhibitor a very useful reagent (38).

Genetic approaches also provide a means of generating specific pathway information regarding mechanism, bearing in mind the potential experimental complications presented in Fig. 3 (column C). Knock-out mice with targeted defects facilitate demonstration of which pathways underlie the associated morphological features of dying cells (71), the potential significance of particular pathways in vivo during infection (89), and the elucidation of an alternate mechanism utilized when the primary mode is blocked (45). Similarly, in systems utilizing transfectable cells, RNA interference can be a tool for identifying key players in pathways leading to cell death (143).

CONCLUSIONS

Despite the widespread use of the apoptosis and necrosis paradigm, a substantial body of literature indicates that the true biological spectrum of cell deaths is much more diverse. Apoptosis is a form of caspase-mediated cell death with particular morphological features and an anti-inflammatory outcome. Necrosis describes the postmortem observation of dead cells that have come to equilibrium with their environment. Oncosis is the prelethal process that occurs in ATP-depleted cells that manifest the morphological changes of swelling and eventual membrane permeability. Autophagy involves degradation of intracellular components within autophagic vacuoles. Pyroptosis is a pathway of cell death that inherently results in inflammation.

Many techniques have been used to measure specific characteristics associated with cell death. Reporting experimental results in terms of the techniques used rather than as percent apoptosis or cell death will clearly indicate the particular fea-

ture of death being measured. By carefully examining the molecular processes that occur in dying cells and paying heed to the outcomes of cell death that influence inflammation and the development of immune responses, we will better characterize novel pathways of cell death and further our understanding of the pathologies underlying a variety of human health problems.

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